C4d Deposits on the Surface of RBCs in Trauma Patients and Interferes With Their Function*

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Objective: Complement system is activated in patients with trauma. Although complement activation is presumed to contribute to organ damage and constitutional symptoms, little is known about the involved mechanisms. Because complement components may deposit on RBCs, we asked whether complement deposits on the surface of RBC in trauma and whether such deposition alters RBC function.

Design: A prospective experimental study.

Setting: Research laboratory.

Subjects: Blood samples collected from 42 trauma patients and 21 healthy donors.

Intervention: None.

Measurements and Main Results: RBC and sera were collected from trauma patients and control donors. RBCs from trauma patients ($n = 40$) were found to display significantly higher amounts of C4d on their surface by flow cytometry compared with RBCs from control ($n = 17$) ($p < 0.01$). Increased amounts of iC3b were found in trauma sera ($n = 27$) (vs 12 controls, $p < 0.01$) by enzyme-linked immunosorbent assay. Incubation of RBC from universal donors (type O, Rh negative) with trauma sera ($n = 10$) promoted C4d deposition on their surface (vs six controls, $p < 0.05$). Complement-decorated RBC ($n = 6$) displayed limited their deformability (vs six controls, $p < 0.05$) in two-dimensional microchannel arrays. Incubation of RBC with trauma sera ($n = 10$) promoted the phosphorylation of band 3, a cytoskeletal protein important for the function of the RBC membrane (vs eight controls, $p < 0.05$), and also accelerated calcium influx ($n = 9$) and enhanced nitric oxide production ($n = 12$) (vs four and eight controls respectively, $p < 0.05$) in flow cytometry.

Conclusions: Our study found the presence of extensive complement activation in trauma patients and presents new evidence in support of the hypothesis that complement activation products deposit on the surface of RBC. Such deposition could limit RBC deformability and promote the production of nitric oxide. Our findings suggest that RBC in trauma patients malfunctions, which may explain organ damage and constitutional symptoms that is not accounted for otherwise by previously known pathophysiologic mechanisms. (*Crit Care Med* 2014; 42:e364–e372)

Key Words: C4d; complement; deformability; nitric oxide; red blood cell; trauma

*See also p. 1323.

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The clinical significance of trauma cannot be underestimated with over 10 million car accidents occurring and over 35 thousand people dying each year in the United States (1). Trauma resulting from accidents or unintentional injuries is the first leading cause of death for those under 50 years old and accounts for one out of about every 20 deaths in the United States (2).

Although trauma usually involves a certain anatomical injury involving extremities or the torso and is followed by obvious pathophysiologic events usually linked to blood loss, it is invariably associated with manifestations from organs not directly affected by injury. The origin of these secondary manifestations is poorly understood. There is information suggesting the presence of an acute inflammatory response in patients experiencing trauma. Specifically, there is evidence that the complement

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system is activated and mediates early posttraumatic inflammatory response (3–5). In addition, the levels of proinflammatory cytokines, including tumor necrosis factor-α, interleukin (IL)-1, and IL-6, are increased in trauma (6, 7). It is suggested that complement activation and proinflammatory cytokine storm occurring in patients with trauma collectively choreograph systemic inflammatory response syndrome associated with acute respiratory distress syndrome and multiple organ failure (8).

In other situations where complement is profusely activated, such as in systemic lupus erythematosus (SLE), complement fragments deposit on the surface of RBCs, which limits their deformability while promoting nitric oxide (NO) production (9). It has been reported that RBC from patients suffering from hemorrhagic shock displays reduced deformability, shows altered morphological appearance, and contributes to the reduction of blood flow to distant organs (10, 11).

Based on the evolving studies, we hypothesized that complement activation affects RBC function in trauma patients. In this communication, we demonstrate that complement becomes activated in trauma patients and split products are deposited on the surface of RBC limiting their ability to pass through capillary-size microchannels and increasing their capacity to produce NO. Our findings suggest that the inhibition of complement can serve as an adjunct treatment in trauma patients.

MATERIALS AND METHODS

Participants

This prospective experimental study was approved by the Institutional Review Board of Beth Israel Deaconess Medical Center and was carried out as anonymized wasted samples under a waiver of consent. A total of 42 patients who visited the emergency department at Beth Israel Deaconess Medical Center for trauma from July 2012 to March 2013 were enrolled nonconsecutively. Trauma patients were 18 years and older and had been admitted for bone fracture, organ injury, or head trauma. They received a blood test as required for their care. Patients carrying the diagnosis of cancer, infection, or autoimmune diseases were excluded. None of the patients had received a transfusion prior to sampling. Healthy donors (n 21) were recruited from the Division of Rheumatology at Beth Israel Deaconess Medical Center after written informed consent. They did not have an apparent illness. RBCs were collected from 40 of 42 trauma patients (two samples were destroyed during the sample collection process) and 17 healthy donors, and sera were collected from 27 patients and 13 healthy donors. Patients' clinical data were recorded, including age, sex, ethnicity, mechanism of injury, and existing injury. Injury Severity Score (ISS) was also calculated.

Antibodies and Reagents

Primary antibodies were anti-C4d monoclonal antibody (A213; Quidel, San Diego, CA) and IgG1k-isotype control (401402; BioLegend, San Diego, CA). Secondary antibody was Alexa Fluor 488-conjugated rabbit anti-mouse IgG (A11059; Life Technologies, Grand Island, NY). Other reagents were as follows: Hanks' balanced salt solution with Ca^{2+} and Mg^{2+} ($HBSS^{++}$) (14025; Life Technologies), IgG-free bovine serum albumin (BSA) (A9085; Sigma-Aldrich, St Louis, MO), 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (D23844; Life Technologies), Flio-4 AM (F14217; Life Technologies), and eosin-5-maleimide (E118; Life Technologies).

Measurement of Serum iC3b Levels

The concentration of iC3b present in serum was measured using the MicroVue iC3b Enzyme Immunoassay kit (A006; Quidel).

RBC Preparation and Incubation With Sera

Heparinized blood or serum of trauma patients was obtained from the emergency department after clinical examination, and control blood or serum was obtained from healthy adult volunteers. The sample collection was performed in accordance with guidelines of the Institutional Review Board of Beth Israel Deaconess Medical Center after informed consent in accordance with the Declaration of Helsinki. Blood was collected in heparin-lithium tubes for RBC and serum in serum-separator tubes. The serum was stored at –80°C. Heparinized blood was washed in $HBSS^{++}$ before use.

For serum studies, on the day of the experiment, the serum samples were thawed from various trauma patients and healthy donors and were used for different measurements under the same conditions. Blood from healthy universal donors (type O, Rh negative) was obtained in heparin-lithium tube and washed with $HBSS^{++}$. One microliter of RBC pellet was resuspended in 80 μL of $HBSS^{++}$, incubated with 20 μL of serum from trauma patients or normal donors for 15 minutes at 37°C, and then washed in $HBSS^{++}$ with 0.5% IgG-free BSA (0.5% BSA/$HBSS^{++}$).

Flow Cytometry

RBCs were incubated for 20 minutes with a primary antibody in 0.5% BSA/$HBSS^{++}$ at room temperature and washed and incubated for another 20 minutes with a secondary antibody directed against the primary antibody at a dilution recommended by the manufacturer. RBCs were then washed and analyzed by FACScan (BD Biosciences, San Jose, CA). At least 10,000 events in each sample were acquired and recorded and analyzed by FlowJo (version 7.6.5 software; Tree Star, Ashland, OR).

Eosin-5-Maleimide Staining

RBCs from healthy universal donors were incubated in the presence of Ca^{2+} and Mg^{2+} with 20% trauma or control sera for 5 minutes at 37°C and then incubated for 15 minutes with eosin-5-maleimide at a final concentration of 0.1 mg/mL. RBCs then were washed three times with $HBSS^{++}$ and 0.5% BSA/$HBSS^{++}$ and analyzed by flow cytometry.

Analysis of Intracellular Calcium Influx

RBCs from healthy universal donors were preloaded with Fluo-4 acetoxymethyl ester (AM) for 15 minutes at 37°C, then washed to remove any dye that is nonspecifically bound with RBC surface, and resuspended in $HBSS^{++}$. Loaded RBCs were incubated at room temperature for an additional 10 minutes to allow complete de-esterification of intracellular AM esters.

Fluorescence levels of RBC were acquired by FACScan for establishing the baseline of intracellular Ca^{2+} concentration. After 60 seconds, 100 μL of control or trauma serum was quickly added to the RBC (20% of final concentration), and the change of the fluorescence intensity that indicates intracellular Ca^{2+} concentration was recorded for an additional 3 minutes.

Measurement of NO Production

RBCs from healthy universal donors preloaded with DAF-FM diacetate in BSA-free $HBSS^{++}$ for 30 minutes at 37°C were washed to remove excess and uncleaved intracellular DAF-FM diacetate probe. Cells were then resuspended in $HBSS^{++}$ with 20% trauma or control serum for 15 minutes at 37°C. RBCs were washed and the fluorescence intensity associated with intracellular NO production was recorded by FACScan using the fluorescence-1 channel.

Measurement of RBC Deformability Using Two-Dimensional Filters

To measure the ability of RBC to undergo capillary-like deformations, we used a two-dimensional microchannel array as previously described (12) that uses the hydrostatic pressure to drive the RBC through the microchannel array. For each experiment, the device was calibrated using as reference the zero pressure difference corresponded to the absence of movement of RBC within the device. RBCs (8 μL, hematocrit 20%) were loaded into the inlet reservoir and driven into the capillary-like area by lowering the waste reservoir tubing. Once RBC entered the capillary-like area, the waste reservoir tubing was raised at a height that allowed RBC to pass through the 25-μm length of the capillary in about 3 seconds. The cells were recorded using a 40×0.75 Ph2 Plan Fluorite objective on a TE300 Nikon-inverted microscope (Nikon, Melville, NY), using a Retiga Exi (QImaging, Surrey, Canada) charge couple device camera controlled with iVision 4.7 software (BioVison, Exton, PA) at a rate of 10 frames/s. Movies representing 600 frames were then analyzed off-line frame by frame, and the results were expressed as the number of seconds from entry to egress (9). RBCs that displayed unusual shapes (e.g., echinocytes and acanthocytes) were excluded from the measurement.

Statistical Analysis

SPSS version 17.0 for Windows (SPSS, Chicago, IL) was used for all statistical analysis. Mann-Whitney *U* test was used for between group analysis and values were showed as medians (interquartile range) unless otherwise stated. Correlation between the ISS and the amount of C4d deposition on trauma RBC was assessed by Spearman correlation coefficient. Paired *t* test analysis was performed wherever necessary. A *p* value of less than 0.05 was considered statistically significant.

RESULTS

Patient Characteristics

A total of 42 patients were included in this study (**Table 1**). The patient group consisted of 33 men and 9 women, with mean age of 47 years. Most of the patients presented with blunt injury resulting from motor vehicle crash (*n* 19) or fall (*n* 16). The median value of ISS was 17 (9–25).

TABLE 1. Demographic and Clinical Characteristics of Research Subjects

Demographic Data	*n* = 42
Age, mean (± SD), yr	46.5 (± 19.6)
Sex (ma e/fema e)	33/9
Ethn c ty, *n* (%)	
Wh te	37 (88)
B ack	2 (5)
H span c	1 (2)
Other	2 (5)
Mechan sm of trauma, *n* (%)	*n* = 42
Motor veh c e crash	19 (45)
Fa	16 (38)
Pedestr an struck	1 (2)
Other	6 (14)
Organ n ury, *n* (%)	
Bone fracture	33 (79)
Head	9 (21)
Lung	7 (17)
Sp een	5 (12)
ntest ne	3 (7)
L ver	0 (0)
K dney	0 (0)
Other	1 (2)
Type of bone fracture, *n*	
Femur	11
Tibia or fibula	10
Sp ne	9
R b	9
Rad us or u na	6
Humerus	3
H p	1
Other	21
Operat ve procedure, *n*	
Orthoped c surgery	23
Thorac c surgery	4
n ury Sever ty Score, mean (± SD)	19.6 (± 12.6)

Deposition of Complement-Activated Product C4d on RBC From Trauma Patients

To determine the extent of complement deposition on the surface of RBC of trauma patients, we used 40 samples from trauma patients and 17 samples from healthy donors. RBC

TABLE 2. Patient Demographics of C4d Deposition on Trauma RBC

Variable	Trauma Patients ($n = 40$)	Healthy Controls ($n = 17$)	p
Age, median (interquartile range), yr	47 (31–50)	36 (34–38)	0.32[a]
Ethnicity, white, n (%)	36 (90)	10 (59)	0.011[b]
Sex, male, n (%)	31 (78)	10 (59)	0.13[b]
Mean fluorescence intensity of C4d level, median (interquartile range)	565 (443–886)	386 (309–489)	< 0.01[a]

[a]Mann-Whitney *U* test.
[b]Fisher exact test.

from trauma patients displayed significant C4d deposition on their membrane as compared with RBC from normal healthy donor (565 [443–886] vs 386 [309–489], $p < 0.01$) (**Table 2**). A representative tracing is shown in **Figure 1*A*** and cumulative data in **Figure 1*B***. We did not find a correlation between the ISS and the amount of C4d deposited on the surface of RBC (data not shown).

It has been reported previously that complement becomes activated in patients who suffer various forms of trauma (4, 13). To confirm that complement activation occurred in our trauma patients, we measured the iC3b levels in the sera obtained from trauma patients and healthy donors. Indeed, iC3b levels were significantly higher in the sera of trauma patients ($n = 27$) compared with sera from healthy donors ($n = 12$) (10.7 [8.2–14.0] vs 6.6 [5.3–9.4], $p < 0.01$) (**Fig. 1*C***).

C4d Deposition on Healthy RBC Incubated With Sera From Trauma Patients

To evaluate the effect of complement deposition on various aspects of RBC function, we first established a method to show that incubation of normal RBC from universal donors (type O, Rh negative) with sera from trauma patients results

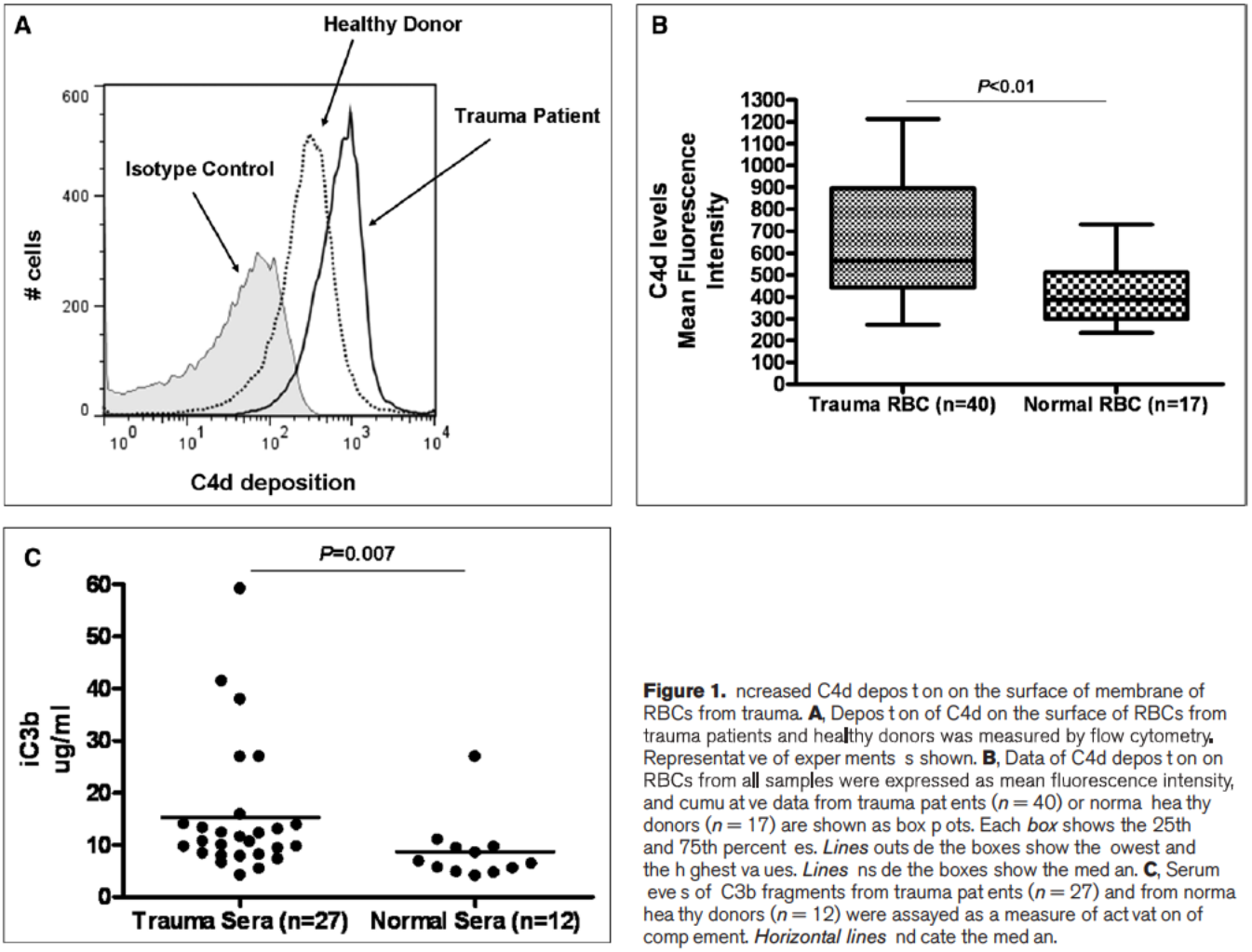


Figure 1. ncreased C4d depos t on on the surface of membrane of RBCs from trauma. **A**, Depos t on of C4d on the surface of RBCs from trauma patients and healthy donors was measured by flow cytometry. Representat ve of exper ments s shown. **B**, Data of C4d depos t on on RBCs from all samples were expressed as mean fluorescence intensity, and cumu at ve data from trauma pat ents ($n = 40$) or norma hea thy donors ($n = 17$) are shown as box p ots. Each *box* shows the 25th and 75th percent es. *Lines* outs de the boxes show the owest and the h ghest va ues. *Lines* ns de the boxes show the med an. **C**, Serum eve s of C3b fragments from trauma pat ents ($n = 27$) and from norma hea thy donors ($n = 12$) were assayed as a measure of act vat on of comp ement. *Horizontal lines* nd cate the med an.

in complement deposition on the surface of RBC. Indeed, as shown in **Figure 2*A*** (representative experiment) and **Figure 2*B*** (cumulative data), incubation of normal RBC with sera from trauma patients (n 10) resulted in a significant C4d deposition on the membrane of RBC as compared with sera from healthy controls (n 6) (289 [282–300] vs 267 [261–272], $p < 0.05$). Removal of Ca^{2+} by adding 10 mM EDTA to the serum decreased the deposition of C4d on the surface of RBC (**Fig. 2*C***), suggesting that activation of complement through the classical pathway accounts for the deposition of complement on RBC (14).

Decreased RBC Membrane Deformability by Trauma Sera

It has been previously reported that complement deposition on RBC can render their membrane less deformable, limiting the ability of RBC to pass through capillaries (15). We evaluated RBC membrane deformability by using a microfluidic device as described in *Materials and Methods* section (**Fig. 3*A***) and measured the time it took RBC to pass through the microchannels. RBCs from a universal donor were treated either with sera from normal individuals (n 6) or trauma patients (n 6). Individual dot blots are presented in **Figure 3*B*** and cumulative data in **Figure 3*C***. Our results show that exposure of RBC to sera from trauma patients limits their ability to pass through capillary-size microchannels (2.03 [1.96–2.25] vs 1.85 [1.81–1.90], $p < 0.05$).

Phosphorylation Status of Band 3

RBC membrane deformability is related to the phosphorylation status of membrane proteins, such as β-spectrin or band 3 (16, 17). To determine whether band 3 becomes phosphorylated in RBC exposed to trauma sera, we treated RBC exposed to control (n 8) or trauma sera (n 10) with eosin-5-maleimide, a reagent which binds to lysine 430 on the extracellular loop of band 3 and is known to indicate tyrosine phosphorylation levels (17). Binding of eosin-5-maleimide to RBC has been used previously to estimate the phosphorylation levels of band 3 (9, 17), and here, we similarly used eosin-5-maleimide to estimate the tyrosine phosphorylation levels of band 3. As shown in **Figure 4**, band 3 of RBC was phosphorylated after incubation with trauma sera compared with incubation with normal sera (89.3 [88.3–92.3] vs 84.8 [83.0–85.5], $p < 0.05$).

Promotion of Ca^{2+} Influx

Tyrosine phosphorylation level of band 3 is known to correlate with intracellular Ca^{2+} concentration (18, 19). We asked

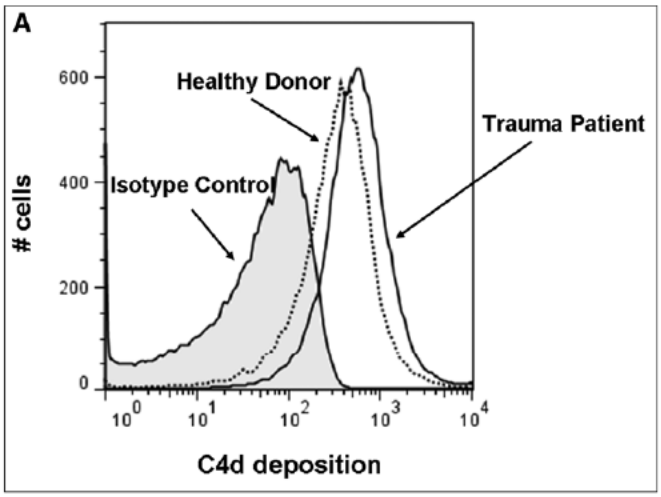
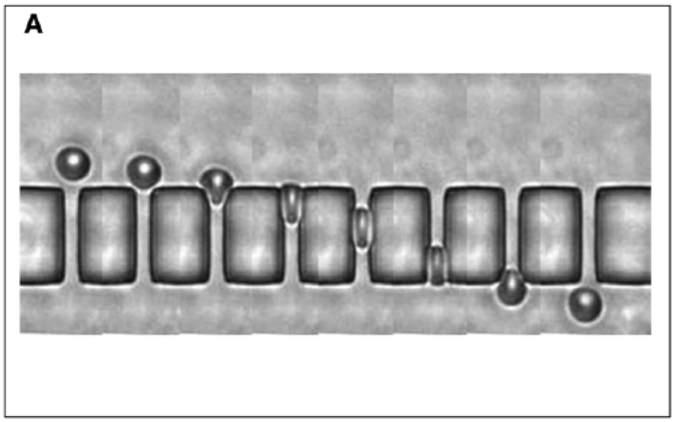
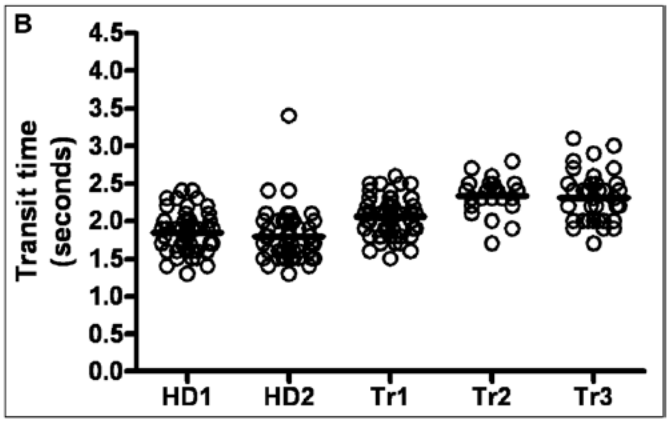


Figure 2. ncreased C4d depos t on on hea thy RBC membranes ncubated w th trauma sera. **A**, Depos t on of C4d on the surface of hea thy RBCs (type O, Rh negat ve) ncubated w th sera from trauma patients or healthy donors was measured by flow cytometry. Representat ve data are shown. **B**, Data of C4d depos t on on RBCs from all samples were expressed as mean fluorescence intensity, and cumu at ve data from trauma pat ents ($n = 10$) or hea thy donors ($n = 6$) are shown as box p ots. Each *box* shows the 25th and 75th percent es. *Lines* outs de the boxes show the owest and the h ghest va ues. *Lines* ns de the boxes show the med an. **C**, Depos t on of C4d on the surface of hea thy RBCs (type O, Rh negat ve) ncubated w th sera from trauma pat ents w th or w thout 10 mM EDTA. Representat ve data are shown.





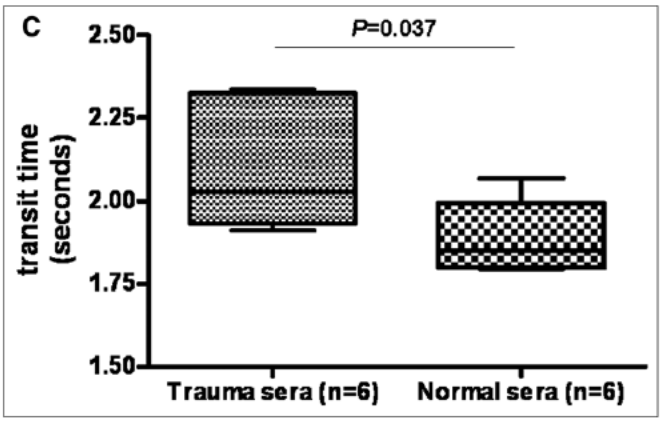


Figure 3. Decreased RBC membrane deformab ty after ncubat on w th trauma sera. **A**, A compos te ser es of mages ustrat ng the passage of a s ng e RBC through a cap ary-s ze m crochanne of the two-dimensional filter device. **B**, ea thy un versa donor's (type O, Rh negat ve) RBCs deformab ty were measured by us ng the two-dimensional filter device after incubation with sera from trauma patients or hea thy donors. Decreased deformab ty of each RBC was assoc ated w th ncreased trans t t me (.e., the t me t took the ce to pass through the cap ary m crochanne s of two-dimensional filter). Data are shown as dot b ots of a exper ments w th each *circle* show ng the passage t me for one RBC. *Horizontal lines* show the mean. Representat ve data are shown. **C**, Cumu at ve data from trauma pat ents ($n = 6$) or hea thy donors ($n = 6$) are shown. Data are expressed as *box* p ots. Each *box* shows the 25th and 75th percent es. *Lines* outs de the boxes show the owest and the h ghest va ues. *Lines* ns de the boxes show the med an. D= hea thy donor, Tr = trauma pat ent.

whether exposure of RBC to trauma sera results in increased Ca^{2+} entry. As shown in **Figure 5*A*** (representative tracings) and **Figure 5*B*** (cumulative data), the addition of trauma sera (n 9) to Fluo-4 AM-loaded RBCs resulted in increased Ca^{2+} concentrations into the RBC compared with RBC to which normal sera (n 4) were added (1.08 [1.05–1.12] vs 1.03 [1.02–1.04], $p < 0.05$).

Induction of the NO Production

Recently, it was reported that human RBCs express an active and functional endothelial-type NO synthase (eNOS), which is present in the membrane and in the cytoplasm (20). In RBC, mechanical stress (shear stress) stimulates NO-generating mechanisms and Ca^{2+} is important in this process (21). Having observed that exposure of RBC to trauma serum results in increased intracellular Ca^{2+} concentrations, we asked whether it also results in an increased NO production. Our results showed that exposure of RBC to trauma sera (n 12) promoted NO production compared with exposure to normal sera (n 8) (543 [527–576] vs 453 [425–501], $p < 0.05$) (**Fig. 6*A***, representative tracing, and **Fig. 6*B***, cumulative data). Increased NO production results from the deposition of complement on the surface of RBC because heat inactivation of sera (55°C for 30 min) eliminated the ability of trauma sera to increase NO production by RBC (**Fig. 6*C***) (597 ± 59 vs 521 ± 21, mean ± SD, paired t test, $p < 0.05$).

DISCUSSION

Complement becomes activated in patients suffering various types of trauma, and this was confirmed in our patients by demonstrating increased levels of iC3b in their sera.

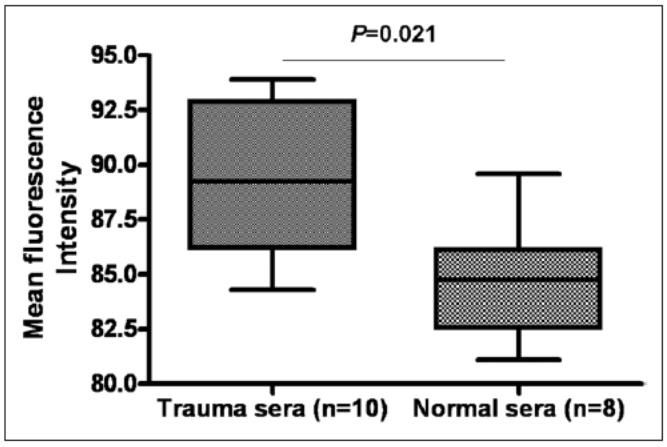


Figure 4. Phosphory at on status of band 3 n RBCs ncubated w th trauma sera. Phosphory at on of band 3 n hea thy RBCs (type O, Rh negative) measured by flow cytometry after incubation with sera from trauma pat ents or hea thy donors, us ng eos n-5-ma e m de sta n ng. Band 3 phosphorylation was expressed as mean fluorescence intensity of eosin-5-maleimide fluorescence, and cumulative data from trauma pat ents ($n = 10$) or hea thy donors ($n = 8$) are shown as box p ots. Each *box* shows the 25th and 75th percent es. *Lines* outs de the boxes show the owest and the h ghest va ues. *Lines* ns de the boxes show the med an.

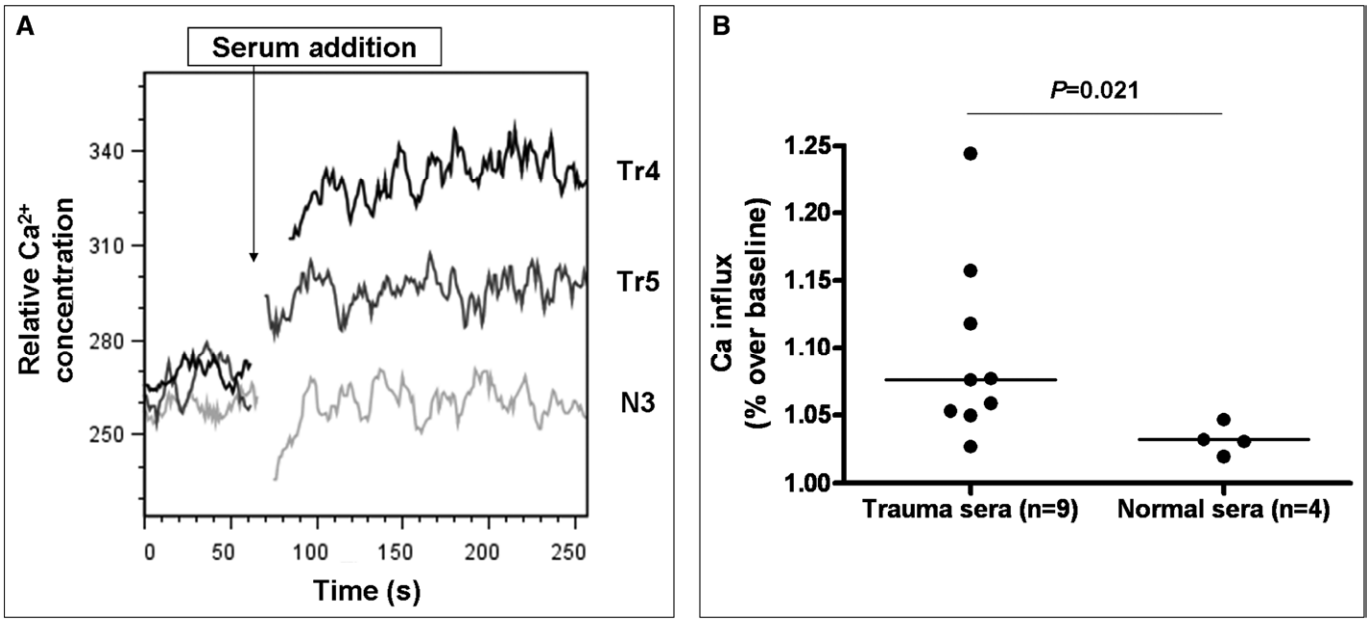


Figure 5. Ca^{2+} influx into RBC after addition of trauma sera. **A**, T me course of serum- nduced changes n ntrace u ar Ca^{2+} of trauma pat ents (Tr4 and Tr5) or a hea thy contro (N3) are shown. *Arrow* nd cates the t me when trauma or contro sera were added to RBC pre oaded w th F uo-4 AM, 1 m n after start of measurement by flow cytometry. *Vertical axis* nd cates re at ve ntrace u ar Ca^{2+} concentration, which is estimated from mean fluorescence intensity of F uo-4 AM. Representat ve data are shown. **B**, Cumu at ve resu ts of Ca^{2+} influx in RBCs to which trauma sera ($n = 9$) or norma sera ($n = 4$) were added. *Vertical axis* shows the ratio of mean fluorescence intensity of Fluo-4 AM before and after adding the serum. *Horizontal lines* nd cate the med an.

Uncontrolled complement activation, common in a wide-variety of conditions both chronic and acute, has multiple deleterious effects on various circulating blood cells and on organ function primarily through the action of inflammatory split products (anaphylatoxins) such as C3a and C5a (22, 23). To date, there are little data regarding the effects of the noninflammatory complement by-products, mainly C3b and C4b generated during complement activation on circulating cell function, specifically RBC. In this study, we show that C4d, a split product of C4, deposits on the surface of RBC, thus limiting their ability to pass through narrow microchannels and enhancing NO production. These functional effects are likely due to the fact that C4d deposition on the surface of RBC promotes a significant RBC Ca^{2+} influx that mediates phosphorylation of band 3.

C4d is the stable remnant of C4, and it has been shown to remain on the surface of RBC in patients with SLE for a prolonged period of time (24, 25). In our study, we demonstrated increased deposition of C4d on the surface of RBC from patients with various degrees and types of trauma; yet, we failed to demonstrate a direct correlation between ISS determined clinically and levels of C4d deposition on the surface of RBC. One possible explanation we were not able to establish the correlation is the logistics involved in handling trauma patients and the collection of blood or sera samples. The time that lapsed between trauma and the collection of samples could not be controlled, which could have affected the overall amounts of C4d that had accumulated on the surface of RBC.

Following the two key observations—the increased complement activation in sera and increased deposition of complement fragment C4d on RBC in trauma patients—we chose to test whether the increase in complement activation and deposition leads to RBC dysfunction. Should binding of complement on the surface of RBC have a functional effect, it should be occurring at early time points. To explore this, RBCs from universal donors were incubated with sera from trauma patients who showed enhanced deposition of C4d on RBC. As noted, exposure of RBC to trauma serum resulted in limitation of their ability to pass through capillaries and increased phosphorylation of band 3, Ca^{2+} entry, and NO production. The alterations in RBC function in trauma patients could be due to deposition of complement fragments on the RBC membranes as others and we have shown both in vitro and ex vivo using RBC from normal individuals (15) and patients with SLE (9).

The functional consequences of the deposition of complement on the surface of RBC cannot be overstated. In order for RBCs to perform their main function, that is to deliver oxygen to tissues, they must pass through narrower (< 8 μm in diameter) capillaries and to do that they have to be able to deform (26, 27). If deposition of C4d renders them difficult to deform and reach tissues, then hypoxia will ensue. During normal conditions, RBC-generated NO is a key factor in the local regulation of vasomotor tone and microvascular flow resistance, by interacting directly with endothelial cells and indirectly with vascular smooth muscles (28). In addition, NO derived from eNOS present in RBC also directly regulates and maintains RBC deformability (20, 29). In our study, we demonstrated that under trauma conditions, increased complement deposition decreases RBC deformability and increases NO production. According to the existing literature, under normal conditions, increase in NO production improves microvascular blood flow. However, in our studies, although the NO production is high in trauma patients,

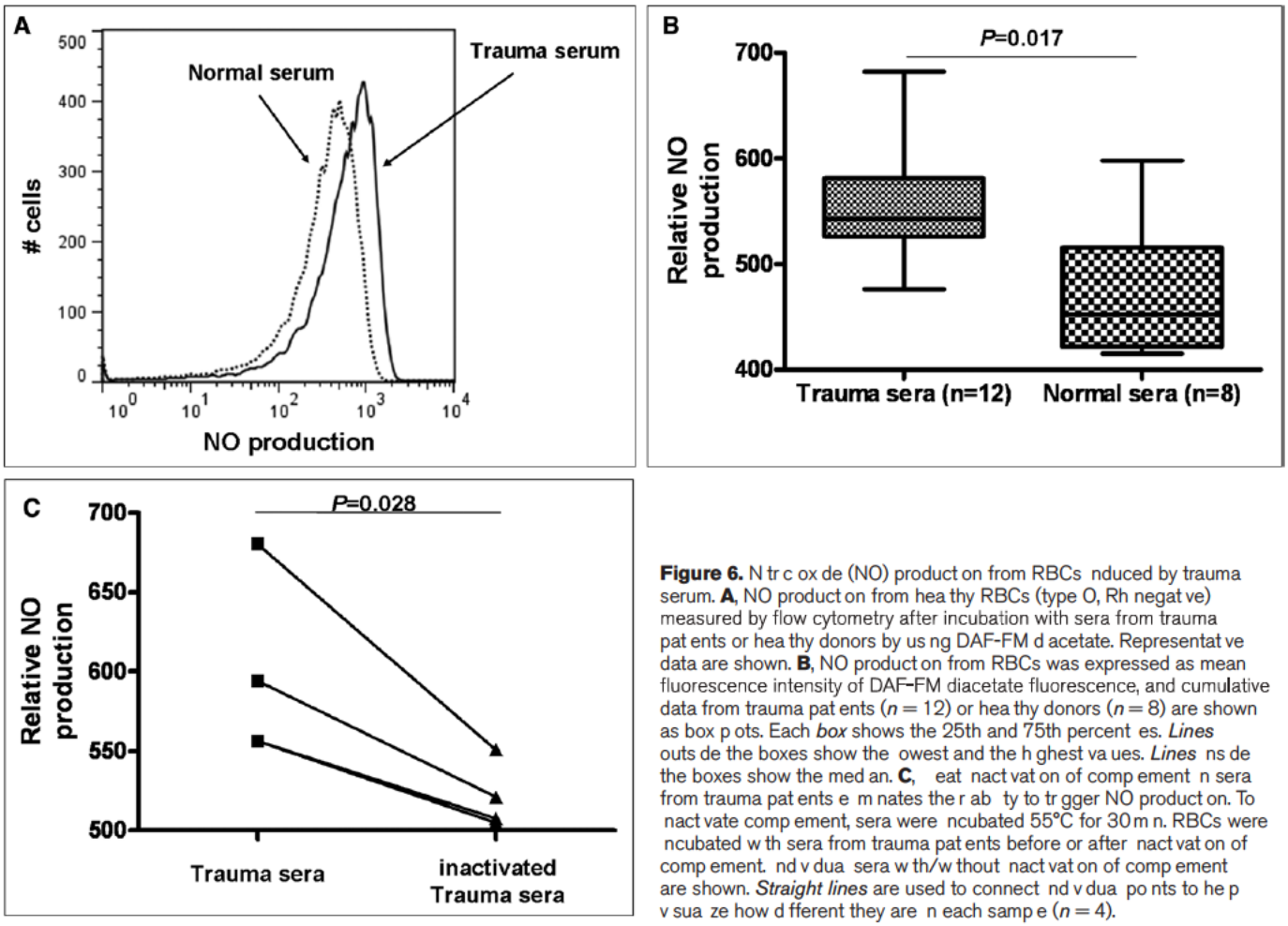


Figure 6. N tr c ox de (NO) product on from RBCs nduced by trauma serum. **A**, NO product on from hea thy RBCs (type O, Rh negat ve) measured by flow cytometry after incubation with sera from trauma pat ents or hea thy donors by us ng DAF-FM d acetate. Representat ve data are shown. **B**, NO product on from RBCs was expressed as mean fluorescence intensity of DAF-FM diacetate fluorescence, and cumulative data from trauma pat ents ($n = 12$) or hea thy donors ($n = 8$) are shown as box p ots. Each *box* shows the 25th and 75th percent es. *Lines* outs de the boxes show the owest and the h ghest va ues. *Lines* ns de the boxes show the med an. **C**, eat nact vat on of comp ement n sera from trauma pat ents e m nates the r ab ty to tr gger NO product on. To nact vate comp ement, sera were ncubated 55°C for 30 m n. RBCs were ncubated w th sera from trauma pat ents before or after nact vat on of comp ement. nd v dua sera w th/w thout nact vat on of comp ement are shown. *Straight lines* are used to connect nd v dua po nts to he p v sua ze how d fferent they are n each samp e ($n = 4$).

we observe a decrease in RBC deformability. We speculate that this may be due to C4d deposition on RBC in trauma patients that may have a prominent effect on RBC deformability compared with NO effect on RBC deformability. The increased production of NO by C4d-decorated RBC in the microcirculation may further alter epithelial and smooth muscle cell function and influence the clinical picture in trauma patients.

Our studies have certain limitations including the fact that the studied cohort was quite diverse. Although we limited the effect of important comorbidities by excluding patients with significant comorbidities from the study, the data about plausible confounding variables, such as smoking, substance abuse, and medications, were not collected. In addition, we were unable to recruit exact age-, ethnicity-, and sex-matched healthy individuals, and hence, these variables show differences between trauma and control groups. Further studies need to be done in clinically homogenous trauma patients compared with matched controls. The second limitation is that although we made every effort to minimize the time between the collection of the blood sample and the performance of the experiments, we could not control the time between the occurrence of traumatic injury and the time the patient was admitted to the emergency department. As indicated above, during that time, variable amounts of C4d could have been deposited on the surface of RBC. Yet, the studies in which RBC from universal donors were exposed to trauma serum collected and stored properly lend validity to our conclusions. A proper prospective study (e.g., collection of serial blood samples from trauma patients during the first hours or days after admission) should be more informative. Third, for the serum-based experiments, instead of whole study sample, we used sera from trauma patients who showed increased C4d deposition on RBC. In this study, our goal was to narrow down our experimental design from an epidemiologic standpoint to a proof-of-concept study to further understand the molecular mechanisms.

CONCLUSIONS

We conclude that C4d decorates the surface of RBC and possibly limits their ability to deform and pass through capillary-size microchannels and increases the production of NO. Thus, it may contribute to trauma-associated morbidity and mortality. As a next step, we plan to conduct a prospective study with larger population, which includes clinically homogenous trauma patients compared with matched controls. We believe that our present and future findings might suggest modalities that limit complement activation in trauma patients to be considered for clinical trials.

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